Effects of Metal Treatment on DNA Repair in Polyamine-depleted HeLa Cells with Special Reference to Nickel

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Human cells depleted of the naturally occurring polyamines putrescine, spermidine, and spermine exhibit altered chromatin structure and marked deficiencies in DNA replicative and repair processes. Similar effects have been observed following treatment of normal mammalian cells with various heavy metal salts. In an attempt to better understand how metals interfere with normal DNA metabolic processes, a series of studies was carried out in which the toxicity and repair-inhibitory properties of various metals were evaluated in polyamine-depleted HeLa cells. Cytotoxicity of copper, zinc, magnesium, and cadmium was not altered in cells carrying lower polyamine pools. However, the sensitivity to nickel was markedly increased upon polyamine depletion, a condition that was readily reversed by polyamine supplementation. Nucleoid sedimentation analysis indicated that a greater amount of nickel-induced DNA damage occurred in polyamine-depleted cells than in normal cells, possibly serving as the basis for the increased sensitivity. Both polyamine depletion and nickel treatment result in decreased repair of DNA strand breaks and decreased cloning efficiency following X-ray and ultraviolet irradiation. Nickel treatment of polyamine-depleted cells resulted in synergistic sensitivity to both radiation treatments. None of the other metals tested enhanced X-ray or ultraviolet sensitivity of polyamine-depleted cells. Analysis of retarded repair sites following ultraviolet irradiation indicated those sites to be nonligatable in polyamine-depleted and nickel-treated cells, suggesting a block in the normal gap-sealing process. — Environ Health Perspect 102(Suppl 3):51–55 (1994).

Key words: nickel, DNA repair, HeLa, polyamines, DFMO, metals, X-ray, UV

Introduction

Considerable evidence now exists suggesting that heavy metals interfere with normal cellular DNA repair processes and that this may result in potentiation of the mutagenic, clastogenic, and carcinogenic effects induced by a variety of agents (*1,2*). Due to the tremendous complexities inherent in cellular metal interactions, the exact nature of this interference is likely to be very difficult to ascertain. However, some clues may be obtained from studies in which repair responses are measured in cells that have been additionally pharmacologically altered. For example, enhanced effects of metals on repair have been observed in human cells depleted of nonprotein thiols (*3*), the largest effects being observed with thiol-reactive metals. These findings are consistent with a number of interpretations and may serve only to underline the probable importance of cellular thiol balance for DNA repair.

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The present study was similarly designed to examine effects of metals in human cells depleted of the naturally occurring polyamines putrescine (PUT), spermidine (SD), and spermine (SP). It has been previously demonstrated that treatment of human cells with α-difluoro-methyl-ornithine (DFMO), an inhibitor of polyamine biosynthesis, depletes cells of PUT and SD, and results in altered chromatin structure (*4*), and deficiencies in repair of both X-ray- (*5*) and ultraviolet-induced (*6*) DNA lesions. Moreover, DFMO-treated mammalian cells also exhibit altered sensitivities to a variety of chemical agents (*7*). The cause of these altered sensitivities is not clear but could relate either to modified chromatin structure or the deficiencies in repair in the polyamine-depleted condition. It was hoped that an examination of the effects of metals in polyamine-depleted cells might help to identify points at which metals interact in their modulation of cellular responses to DNA damaging insults.

Materials and Methods

Cell Culture, Metal Treatment, and Polyamine Depletion

Human HeLa cells were cultured in minimal essential medium containing 10% fetal bovine serum at 5% CO_2 and 37°C. Cultures were depleted of cellular polyamines by treatment with DFMO (Marion Merrell Dow, Cincinnati, OH) for 48 hr, 2.5 mM with or without the addition during the last 24 hr of a second inhibitor of polyamine biosynthesis, MDL 73,811 (50 μM) (Marion Merrell Dow). Metal salts (reagent grade) were made up as 100× stock solutions in HEPES/glucose buffer (50 mM HEPES, 100 mM NaCl, 5 mM KCl, 5 mM glucose, 2 mM $CaCl_2$, pH 7.2). For all experiments, medium was removed from dishes and cultures were treated with metal in buffer for 2 hr. Nonmetal-treated cultures received HEPES/glucose for 2 hr. The 2-hr treatment of cells with buffer alone did not affect viability or cloning efficiency. Metal salts employed in this study were $NiCl_2 \cdot 6H_2O$, $CdCl_2 \cdot 2.5\ H_2O$, $CuCl_2 \cdot 2H_2O$, $ZnCl_2$, and $MgCl_2 \cdot 6H_2O$.

Colony-Forming Ability

The ability of HeLa cells to form colonies was assessed by reseeding appropriately treated cultures at 500 to 1000 cells per dish, allowing 7 days growth, methanol fixation, and Dif-Kwik (Baxter Healthcare) staining of dishes for colony counting. Untreated HeLa cells exhibit approximately 25 to 40% colony-forming ability whereas DFMO and DFMO/MDL

Table 1. Effects of polyamine biosynthesis inhibitors on cellular polyamine pools.

Treatment	Putrescine, %[a]	Spermidine,%	Spermine, %
Control	100	100	100
DFMO[b]	4±3	16±7	98±14
DFMO/MDL 73,811[c]	7±11	22±3	38±20

[a] Polyamine levels in untreated control cells were 725 ± 110, 3250 ± 635 and 4080 ± 229 pmole/10^6 cells for putrescine, spermidine, and spermine, respectively. [b] 2.5 mM, 48 hr. [c] 2.5 mM DFMO, 48 hr, 10 μM MDL 73,811, last 24 hr.

Table 2. Effects of metal treatment on colony-forming ability of normal and polyamine depleted HeLa cells.

	Approximate IC_{50}, μM[a]		
	Control	DFMO[b]	DFMO/NDL 73,811[c]
Cu^{2+}	30	30	30
Zn^{2+}	30	20	25
Mg^{2+}	>10,000	>10,000	>10,000
Cd^{2+}	28	22	35
Ni^{2+}	920	77	85

[a] Two-hour treatment of cultures in HEPES/glucose buffer with or without metal salt. Cells immediately harvested and replated at clonal density for determination of colony-forming ability. [b] 2.5 mM, 48 hr. [c] 2.5 mM DFMO, 48 hr/10 μM MDL 73,811, last 24 hr.

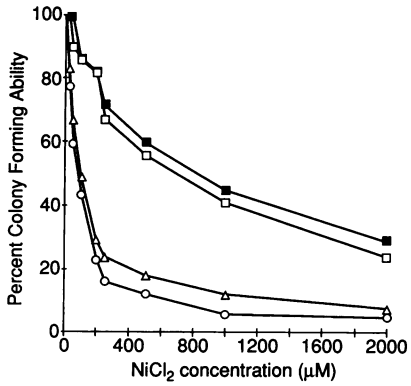

Figure 1. Effect of nickel on colony-forming ability of polyamine-depleted HeLa cells. Untreated (■) or DFMO-treated (○) cultures were exposed to nickel for 2 hr in HEPES/glucose buffer and immediately harvested and reseeded at clonal density for colony formation. In some cases, 1 mM putrescine was added to DFMO-treated cultures for 3 hr prior to nickel treatment (□) or at the time of reseeding (△). Results are averages of three to seven determinations. Error was less than 10% for all determinations.

73,811-treated cultures clone at 25 and 5% respectively.

Polyamine Analysis

Cellular polyamine levels were measured by reversed phase HPLC as described previously (*8*).

Irradiation

X-irradiation of cultures was carried out in complete growth medium in a TFI Bigshot X-ray unit (TFI Corp., West Haven, CT) run at 3 mA, 50 KV, 1.5 mm Be filtration, and generating 1.6 Gy/min. Ultraviolet irradiation was carried out on medium using a GE germicidal lamp emitting 1.2 J/M^2/sec at 19 in. All media were removed during UV irradiation.

Nucleoid Sedimentation Analysis of DNA Strand Breaks and Repair

Nucleoid sedimentation was performed basically as described by Cook and Brazell (*9*) with modifications described in detail elsewhere (*3*).

Structure of Repairing Sites

To determine the molecular structure of aborted repairing sites, a modification of the procedure of Cleaver (*10*) was employed as described previously (*6*). Basically, the assay measures the ability of exonuclease III to remove repair-incorporated [^{3}H]-bromodeoxyuridine from purified cellular DNA following treatment of that DNA with T4 DNA ligase. See footnotes to Table 5 for details.

Results

Inhibition of the intracellular polyamine biosynthetic enzymes, ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) by α-difluoromethylornithine (DFMO) (*11*) and MDL 73,811 (*12*), respectively, results in a predictable reduction in polyamine pools. Table 1 demonstrates that DFMO treatment markedly reduces PUT and SD but has little or no effect on SP pools. All three polyamines can be reduced by combined inhibitor treatment. These effects have been well studied and the above results are consistent entirely with what is known regarding modulation of cellular polyamine levels with these inhibitors.

It was discovered that polyamine-depleted HeLa cells exhibited increased sensitivity to killing by $NiCl_2$, whereas sensitivity to Cu^{2+}, Zn^{2+}, Mg^{2+}, and Cd^{2+} was not markedly affected (Table 2). Since the Ni^{2+} sensitivity of double inhibitor-treated cultures was similar to that of DFMO-treated cultures, all subsequent studies involving colony-forming ability were conducted with DFMO-treated cultures. As shown in Figure 1, a 3-hr treatment with 1 mM PUT prior to Ni^{2+} exposure completely restored normal sensitivity to Ni^{2+}, whereas PUT addition after Ni^{2+} exposure (at the time of reseeding) had no such protective effect. The basis for the increased sensitivity in polyamine-depleted cells appears to be an increased amount of Ni^{2+}-induced DNA damage. Table 3 demonstrates that DNA single-strand breaks are seen in control HeLa cells only after exposure to about 225 μM $NiCl_2$. In contrast, breaks are

Table 3. DNA strand breaks induced by nickel treatment in normal and polyamine-depleted HeLa cells and their rates of repair.

$NiCl_2$ Dose (μM)[a]	25	77	225	450	920	T_{50}[b]	T_{75}[b]
Control	0	0	40[c]	80	200±40	2.6	3.9
DFMO	110±30	215±20	>300	>300	>300	2.8	4.0
DFMO/73,811	ND	240±30	280	>300	>300	ND	ND

ND=not determined. [a] HeLa cells were treated for 2 hr with metal in HEPES/glucose buffer at the indicated dose and immediately harvested for DNA strand-break analysis by nucleoid sedimentation. [b] Time in hours required for return of nucleoids to 50 and 75% normal sedimentation position in gradients. Controls and DFMO-treated cultures were treated with 450 and 20 μM $NiCl_2$, respectively. [c] X-ray doses from 20 to 300 rads produce a linear decrease in sedimentation of nucleoids through neutral sucrose gradients (*5*), allowing calibration of metal-induced DNA breaks in rad equivalents as presented.

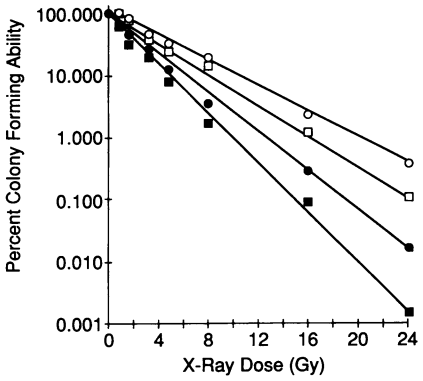

Figure 2. Effect of nickel on X-ray sensitivity of polyamine-depleted HeLa cells. Untreated (○), DFMO-treated (●), nickel-treated for 2 hr at 1 mM in HEPES/glucose (□), or DFMO/80 μM nickel-treated (■) cells were X-irradiated over an appropriate dose range and immediately reseeded at clonal density in the absence of drug or metal for colony-forming ability. Control and DFMO data are derived from at least ten experiments; nickel and DFMO/nickel data are the means of three determinations.

clearly observed in polyamine-depleted cells following doses as low as 25 μM $NiCl_2$ (lower doses were not examined). Since, in these studies, cells are analyzed for DNA breaks after extended exposure to metal, breaks observed result both from direct Ni^{2+}-induced DNA damage and as a consequence of the excision repair process operating at those sites. To determine if the apparent increased damage was due to retarded break resealing, repair was monitored as a function of time. Table 3 shows that following $NiCl_2$ exposures that induced roughly the same amount of damage, the rate of disappearance of breaks was similar in untreated and DFMO-treated HeLa cells. Thus it is likely that the elevation of single-strand breaks was due to increased yield of damage in polyamine-depleted cells.

Polyamine-depleted HeLa cells exhibit increased radiosensitivity relative to normal cells (Figure 2). The D_0 (the reciprocal of the slope of the terminal linear region of the survival curve) was 4.25 and 2.8 Gy for control and DFMO-treated cultures, respectively. This translates to a reduction in the X-ray IC_{50} for killing from 3.72 Gy to 1.93 Gy. HeLa cells treated for 2 hr with 1 mM $NiCl_2$ also showed enhanced radiosensitivity ($D_0 = 3.50$ Gy, X-ray $IC_{50} = 3.10$ Gy). Treatment of DFMO-treated cultures with 80 μM $NiCl_2$ for 2 hr (a metal dose that resulted in equivalent lowering of colony forming ability to that of nonpolyamine-depleted cells) resulted in an even greater radiosensitivity ($D_0 = 2.07$ Gy, X-ray $IC_{50} = 1.51$ Gy).

Polyamine-depleted cells have also been shown to exhibit retarded sealing of X-ray-induced DNA strand breaks (*5*). Figure 3 demonstrates that this inhibition is very apparent at short times after irradiation and that by 4 hr most breaks are sealed, as suggested by the return of nucleoids to the nonirradiated control position in the gradient. We have also previously reported that repair of X-ray breaks is retarded in cells treated with $NiCl_2$ (*13*). To reexamine this in DFMO-treated cells, $NiCl_2$ doses were chosen to minimize the background of DNA breaks induced by metal treatment alone. Thus, control and DFMO-treated cells were exposed for 2 hr to 200 and 25 μM $NiCl_2$, respectively, prior to irradiation. Nucleoid sedimentation under these conditions was nearly normal and was used as the baseline for all subsequent X-ray studies. Figure 3 shows that Ni^{2+} alone has weak activity in retarding repair but appears synergistic in this regard in DFMO-treated cells with only 20% recovery seen at 4 hr postirradiation. Under these treatment conditions and X-ray dose (16 Gy) CFA was 4, 4, 0.5, and 0.09% for control, control+Ni^{2+}, DFMO, and DFMO+Ni^{2+}, respectively. These studies suggest that Ni^{2+}-induced radiosensitivity may be due to a potentiation of the already weakened repair response of polyamine-depleted cells.

DFMO-treated cells are also sensitive to UV irradiation (*6*). In the present studies, the UV dose required for 90% killing was reduced from 30 to 19 J/M² in DFMO-treated cells (Table 4). Nickel did not markedly enhance UV killing of either control or DFMO-treated cells when administered at its IC_{50} dose in each case. Cells allowed to repair for 1 hr following irradiation exhibit repair-dependent DNA breaks which are greatly enhanced when repair occurs in the presence of repair inhibitors such as *ara*-C (Table 4). Both DFMO- and nickel-treated cultures exhibit more such breaks than controls and additivity is seen upon combined treatment. Thus, UV repair appears to be affected similarly to X-ray repair by Ni^{2+} treatment.

Cleaver (*10*) developed an assay for probing both the completeness of excision repair and the structure of aborted or retarded repairing sites. In that assay, cellular DNA containing repair-incorporated [^{3}H] bromodeoxyuridine was purified from isopycnic cesium chloride gradients and digested with exonuclease III. Radioactivity released was assumed to be at sites that had not completed repair. If extensive DNA ligase treatment prior to exonuclease digestion reduced the amount of released radiolabel, it was concluded that the incomplete sites were capable of being ligated. We used this assay previously to demonstrate that retarded repairing sites in DFMO-treated cells were not ligatable, i.e., gaps rather than nicks. Table 5 confirms these results and extends the findings to nickel treatment. Exonuclease III releases radiolabel from UV-irradiated Ni^{2+}-treated cells and T4 DNA ligase does not reduce this. Consistent with the UV repair data above, nickel treatment of polyamine-depleted cells leads to further increased release of radiolabel. It is concluded from these studies that Ni^{2+}-treatment impedes repair most likely through inhibition of the gap sealing process.

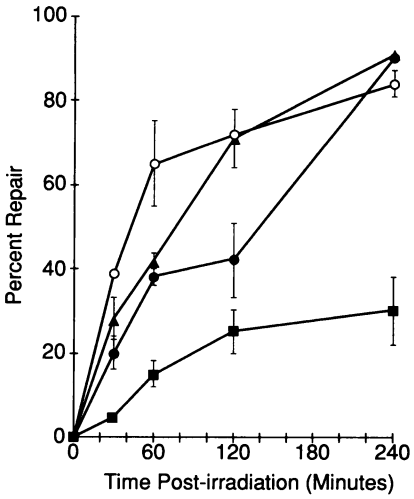

Figure 3. Effect of nickel on the resealing of X-ray induced DNA single-strand breaks. Cultures were irradiated with 16 Gy X-rays and either harvested immediately or incubated in fresh nondrug/metal containing media for the appropriate periods of time prior to harvesting and analysis of DNA strand breaks by nucleoid sedimentation. Percent repair refers to the restoration of the sedimentation of nucleoids to their unirradiated position in the gradient. Untreated control (○), DFMO-treated (●), 200 μM $NiCl_2$, 2hr HEPES/glucose (▲), DFMO/ 25 μM $NiCl_2$, 2hr HEPES/glucose (■). Data are single determinations except where error bars designate the mean ± SE of three to nine separate determinations.

Discussion

The present studies indicate that HeLa cells depleted of PUT and SD are hypersensitive to killing by Ni^{2+} but not by several other divalent metal cations. This sensitivity is not augmented by additional inhibitor treatments that also reduce cellular SP pools, suggesting that SP may not play a role in modulating sensitivity to nickel. Putrescine supplementation readily restores nickel sensitivity. In the polyamine-depleted state, Ni^{2+} apparently induces more DNA damage (Table 3), which probably accounts for

Table 4. Effects of nickel treatment on sensitivity to ultraviolet light in polyamine-depleted HeLa cells.

Treatment	UV IC_{90} (J/M^2)[a]	Rad equivalent DNA breaks[b]
Control	30	60
DFMO	19	125
$NiCl_2$, 80 μM	28	80
$NiCl_2$, 200 μM	26	150
DFMO/25 μM $NiCl_2$	ND	135
DFMO/80 μM $NiCl_2$	16	240
ara-C, 20 μM	ND	290

ND = not determined. Cultures were irradiated with 2.0 J/M^2 UV_{254} and incubated for 1 hr in fresh medium containing no inhibitors (except in case of *ara*-C, in which inhibitor was added only during postirradiation incubation). After incubation, cells were harvested and analyzed for DNA strand breaks by the nucleoid sedimentation assay. [a]UV dose which reduces cloning efficiency by 90%. [b]Rad equivalent breaks calculated from nucleoid position in gradients after correction, where necessary, for nickel-induced breaks.

Table 5. Ligase sensitivity of retarded repairing sites in nickel-treated UV-irradiated HeLa cells.

Treatment	^3H released by exonuclease III[a] (cpm) (–)Ligase	(+)Ligase[b]
Control	147 (3)[c]	211 (4)
Control plus *ara*-C	1490 (31)	1950 (37)
DFMO	1114 (23)	1226 (24)
Nickel, 920 μM; 2 hr pre-UV	1080 (21)	950 (22)
Nickel, 77 μM; 2 hr pre UV	215 (6)	209 (8)
DFMO/ 77 μM nickel	2424 (42)	2618 (48)

Cultures were irradiated with 20.0 J/M^2 of UV_{254} light and allowed to repair in the presence of 4 μCi/dish [^3H] bromodeoxyuridine (Amersham, 40 Ci/mmole). Parental density DNA from 1×10^7 cells was recovered from neutral cesium chloride density gradients. For enzyme probing, approximately 5000 cpm of repair-incorporated DNA was used per reaction tube. [a]*E. coli* exonuclease III added at 75 u/≈μg DNA, 30 min, 37°C. [b]T4 DNA ligase added at 50 u/μg DNA, 30 min, 37°C. [c]Number in parentheses is percent of total repair-incorporated label.

the increased sensitivity. Increased DNA damage is also readily reversible by PUT addition (not shown). There are at least two explanations for this increased DNA damage. First, nickel is known to complex with amines (*14*) and it is possible that removal of millimolar concentrations of polyamines allows more free Ni^{2+} to enter the nucleus. Alternatively, chromatin structural alterations associated with polyamine depletion (*4*) may make the DNA more accessible to nickel ion. Costa et al. have demonstrated a dependency for Ni^{2+} binding to DNA on chromatin structure (*15–17*). Although the exact nature of the chromatin changes associated with DFMO treatment are not known, it is not unreasonable to assume that they might allow for greater nickel interactions with DNA.

Inhibition of DNA repair processes by nickel has been previously reported (*13,18–20*) but little is known of the nature of this effect. Thiol depletion in HeLa cells enhanced nickel toxicity approximately 40-fold but did not markedly enhance the radiosensitization of those cells by nickel (*3*). In contrast, polyamine depletion enhanced nickel toxicity about 10-fold (Figure 1) and also resulted in apparent synergistic effects on X-ray repair (Table 3; Figures 2,3) and at least additive effects on UV repair (Table 4). The present studies do not allow a determination of how polyamine depletion accentuates the repair inhibitory properties of nickel. As argued above, however, it is possible that more uncomplexed Ni^{2+} is available for interaction with cellular macromolecules or repair enzymes. The recent finding by Hartwig et al. (*20*) that Mg^{2+} antagonizes the repair inhibitory effects of Ni^{2+} is consistent with the notion that metals might act through altering the catalytic function of repair enzymes. Polyamines may serve many of the same cellular functions as Mg^{2+} and have been shown to stimulate repair enzymes (*21*), presumably through stabilization of enzyme/DNA complexes. A likely scenario, then, is one in which repair enzymes have difficulty in interacting with DNA due to the deficiency of cellular polyamines following DFMO treatment. These enzymes are then further susceptible to interaction with other cations, e.g., Ni^{2+}, which may additionally compromise their catalytic functions.

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